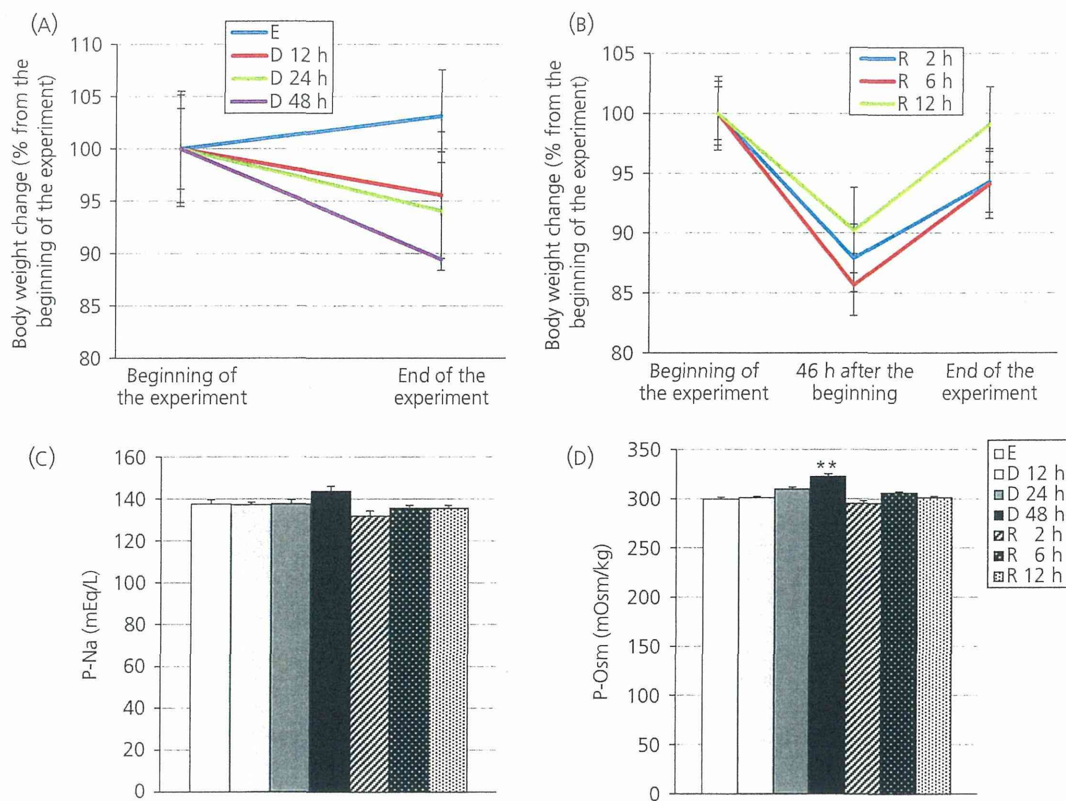

Fig. 5. The body weight change and plasma measurements in female rats. (A) Body weight changes on euhydration and 12, 24 and 48 h of dehydration. (B) Body weight changes on 2, 6 and 12 h of rehydration. All data are presented as percentages from the beginning of the experiments. (C) Plasma sodium concentration (P-Na) in male rats, (D) Plasma osmolality (P-Osm) in male rats. E, *ad lib.* to water; D 12 h, water deprivation for 12 h; D 24 h, water deprivation for 24 h; D 48 h, water deprivation for 48 h; R 2 h, water deprivation for 46 h + *ad lib.* to water for 2 h; R 6 h, water deprivation for 46 h + *ad lib.* to water for 6 h; R 12 h, water deprivation for 46 h + *ad lib.* to water for 12 h. **$P < 0.01$ versus all other groups. All data are presented as the mean ± SEM.

cells increase in the AP (Figs 7E-a and 8) and the NTS (Figs 7E-b and 8).

In the RVLM, the number of mRFP1 positive cells following 2 h of rehydration (Fig. 7E-c) were not statistically changed compared to dehydration (Fig. 8). After 6 h of rehydration, basal, euhydrated levels of mRFP1 expression were restored in all brainstem regions (Figs 7F-a–F-c and 8) and this low level of expression was maintained to the 12-h rehydration time point (Figs 7G-a–G-c and 8). Similar results were seen in female rats, with no significant differences identified between the sexes.

Expression patterns of c-Fos-LI positive cells in the brain and brainstem

Expression of c-Fos-LI cells in male and female rats was observed in the OVLT, MnPO, SFO, SON, PVN, AP, NTS and RVLM in euhydrated rats, 48 h dehydrated rats, and in rats dehydrated for 46 h then rehydrated for 2, 6 or 12 h (Fig. 9). Quantification of transgene expression involved counting the number of c-Fos-LI positive cells. In all the regions examined, very few c-Fos-LI positive cells were seen in the euhydrated state (Fig. 9A-a–A-h, D-a–D-h). Dehydration resulted in a significant increase in the number of c-Fos-LI positive cells in all the observed regions (Fig. 9B-a–B-h, E-a–E-h). Then the number of the cells returned to the euhydrated level after 12 h of rehydration (Fig. 9C-a–C-h, F-a–F-h). There were no significant differences identified between the sexes.

Discussion

The present study has revealed the effects of chronic osmotic stimulation and subsequent water rehydration on mRFP1 fluorescence in specific forebrain and the brainstem regions of male and female c-*fos*-mRFP1 transgenic rats. We observed strong mRFP1 signals after dehydration in all the osmosensitive areas, as previously reported in Fos immunostaining studies (8–10). This may suggest that the mRFP1 signals have at least equal sensitivity or more to immunostaining for Fos protein. However, the assessment of mRFP1 expression is relatively straightforward, and acts as a facile and sensitive proxy by which to quantify neuronal activity in osmosensitive neurones.

In the male and female forebrain SON and PVN, the hindbrain RVLM, and in the OVLT (a forebrain circumventricular organ, CVO), 12 h of dehydration resulted in a massive and significant increase in the number of mRFP1 positive cells, and this did not change at

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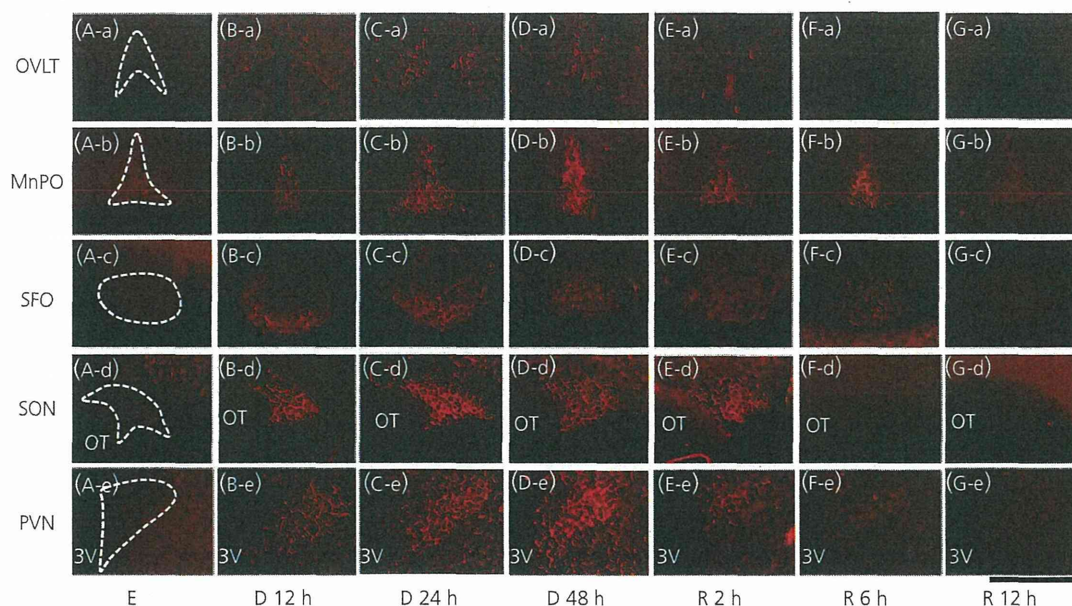

Fig. 6. Digital images of representative examples of monomeric red fluorescent protein 1 (mRFP1) expression patterns in the forebrain in female rats. The white dotted lines show the location of analysis (A-a–A-e). The conditions used in these studies are shown at the bottom of each vertical column, and the brain areas being assayed are shown on the left. E, *ad lib.* to water; D 12 h, water deprivation for 12 h; D 24 h, water deprivation for 24 h; D 48 h, water deprivation for 48 h; R 2 h, water deprivation for 46 h + *ad lib.* to water for 2 h; R 6 h, water deprivation for 46 h + *ad lib.* to water for 6 h; R 12 h, water deprivation for 46 h + *ad lib.* to water for 12 h; OT, optic tract; 3V, third ventricular; OVLT, organum vasculosum of the lamina terminalis; MnPO, median preoptic nucleus; SFO, subfornical organ; SON, supraoptic nucleus; PVN, paraventricular nucleus. Scale bar = 500 μm.

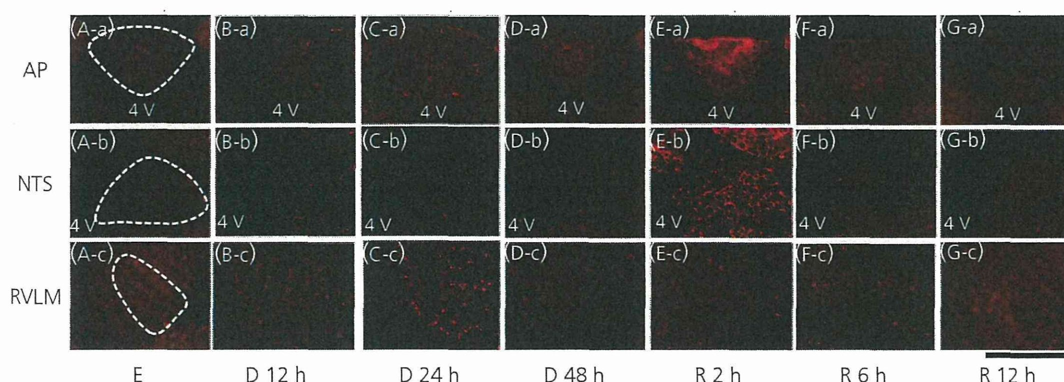

Fig. 7. Digital images of representative examples of monomeric red fluorescent protein 1 (mRFP1) expression patterns in the brainstem in female rats. The white dotted lines show the location of analysis (A-a–A-c). The conditions used in these studies are shown at the bottom of each vertical column, and the brain areas being assayed are shown on the left. E, *ad lib.* to water; D 12 h, water deprivation for 12 h; D 24 h, water deprivation for 24 h; D 48 h, water deprivation for 48 h; R 2 h, water deprivation for 46 h + *ad lib.* to water for 2 h; R 6 h, water deprivation for 46 h + *ad lib.* to water for 6 h; R 12 h, water deprivation for 46 h + *ad lib.* to water for 12 h; 4V, fourth ventricular; AP, area postrema; NTS, nucleus of the solitary tract; RVLM, rostral ventrolateral medulla. Scale bar = 500 μm.

24 or 48 h of dehydration. Thus, regardless of the degree of progressive bodily water depletion, there is no change in the number of mRFP1 positive cells beyond the initial induction over the first 12 h of the stimulus. This is also the case for the female forebrain OVLT CVO. However, in contrast, progressive dehydration induced a gradual increase in mRFP1 positive cells in the male MnPO and SFO forebrain CVOs. The physiological significance of this sexual dimorphism remains to be determined. Note that we studied randomly cycling female transgenic rats, and we concede that oestrous cycle differences may skew our results to an unknown extent. Several studies report that there are oestrogen receptors in the MnPO and SFO (11,12). Oestrogen may attenuate Fos induction in the MnPO and SFO after physiological stimuli such as dehydration.

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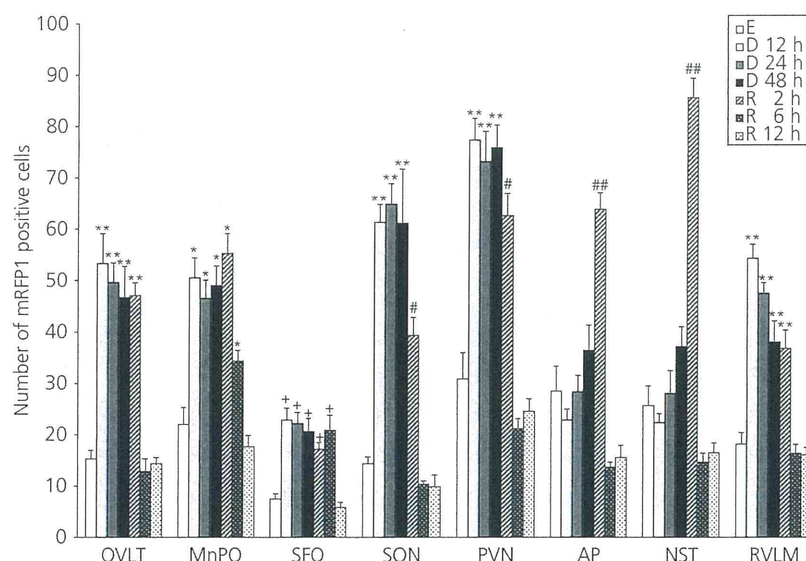

Fig. 8. Changes in number of monomeric red fluorescent protein 1 (mRFP1) positive cells in the forebrain and brainstem in female rats. E, *ad lib.* to water (n = 6); D 12 h, water deprivation for 12 h (n = 6); D 24 h, water deprivation for 24 h (n = 6); D 48 h, water deprivation for 48 h (n = 7); R 2 h, water deprivation for 46 h + *ad lib.* to water for 2 h (n = 12); R 6 h, water deprivation for 46 h + *ad lib.* to water for 6 h (n = 6); R 12 h, water deprivation for 46 h + *ad lib.* to water for 12 h (n = 6). *P < 0.05 versus E and R 12 h. **P < 0.01 versus E, R 6 h and R 12 h. ⁺P < 0.05 versus E and R 12 h. #P < 0.05 versus E, R 6 h and R 12 h. ##P < 0.01 versus all other groups. OVLT, organum vasculosum of the lamina terminalis; MnPO, median preoptic nucleus; SFO, subfornical organ; SON, supraoptic nucleus; PVN, paraventricular nucleus; AP, area postrema; NTS, nucleus of the solitary tract; RVLM, rostral ventrolateral medulla. Data are presented as the mean ± SEM.

Strikingly, dehydration had no inductive effect on mRFP1 expression in the NTS. Gottlieb *et al.*(8) have reported an increase in *c-fos* in the NTS and AP after 48 h dehydration, which is consistent with our c-Fos-LI study (Fig. 9). The rats that they used were Sprague-Dawley rats (250–350 g body weight). It is likely that strain and weight differences contribute to the differences between their experiments and ours. For example, water deprivation for 48 h may develop different degrees of hypovolemia, which resulted in differential mRFP1 expression in the NTS and AP (14).

Similarly, and in contrast to the forebrain CVOs, including the osmosensitive SFO, the AP, a hindbrain CVO, does not up-regulate mRFP1 as a consequence of chronic dehydration. We have previously used Affymetrix microarray expression profiling (Affymetrix, Santa Clara, CA, USA) to compare those genes that are significantly regulated by chronic (72 h) dehydration in the SFO (15) and AP (16). Interesting, many more genes (305) were regulated in the SFO compared to the AP (53), suggesting that the SFO is much more sensitive to chronic water deprivation that the AP. Furthermore, the genes regulated by chronic dehydration in the SFO may be transcriptional targets of *c-fos*.

As well as documenting the effects of chronic dehydration on mRFP1 expression in the brains of our transgenic rats, we also investigated the consequences of 2, 6 and 12 h of rehydration. Strikingly, and in marked contrast to the lack of responses to chronic dehydration, 2 h of rehydration induced a massive but transient increase in mRFP1 expression in the AP and NTS; by 6 h, expression was back to basal levels. The NTS is involved in gustatory processing (17) and we speculate that the NTS shows increases in mRFP1 positive cells associated with water intake. The NTS receives afferent projections from other brain regions and a variety of organ systems (6,18), which may also play a role in the expression of mRFP1 after rehydration. The privileged location of the AP outside of the blood–brain barrier makes this sensory circumventricular organ a vital player in the control of autonomic functions by the central nervous system (19). It has been described that the AP is involved in nausea and vomiting (20). Although details of the mechanism are unknown, it may be presumed that the AP responds by nausea that occurs after rehydration.

After 2 h of rehydration, the number of mRFP1 positive cells in the OVLT, MnPO and SFO was as same as in the dehydrated state. These three forebrain CVOs have been described to contain osmosensitive neurones that project to the SON and PVN, and they play an important role in cardiovascular regulation and body fluid homeostasis (5,21,22). It has been suggested that Fos immunostaining in the OVLT and MnPO after rehydration is a result of sustained activation of the renin–angiotensin system (23).

The number of mRFP1 positive cells returned to the euhydrated state in the SON and PVN after 6 h of rehydration, and were attenuated in the OVLT and MnPO, then completely returned to the basal euhydrated levels after 12 h of rehydration in all the areas we observed. Previous studies suggested that the number of cells that were immunostained for Fos in the SON returned to the euhydrated levels after only 2 h of water intake (8). It is thus possible that our mRFP1 Fos-surrogate has a different half-life to the native Fos protein, or that our transgene is subject to marginally different regulatory control.

Previous studies have revealed that the MnPO is involved noradrenergic control of body fluid volume, and it has been suggested

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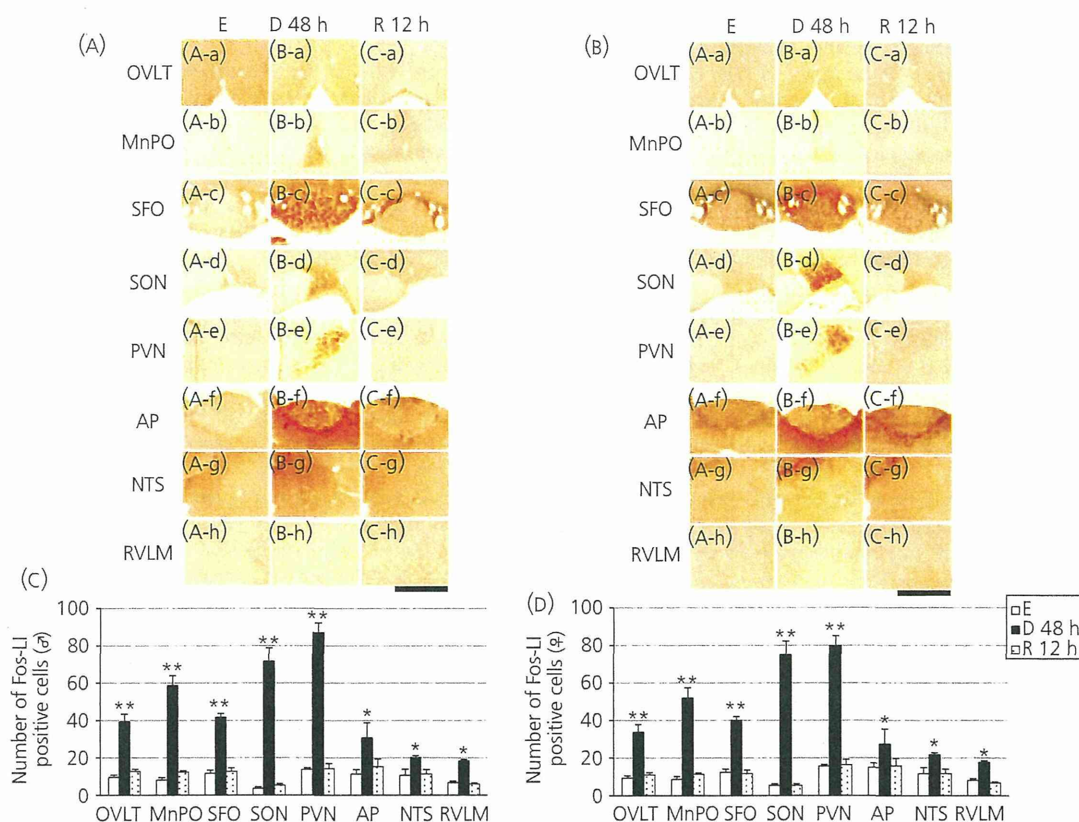

Fig. 9. Immunostaining for Fos protein in the brain and brainstem. (A) Digital images representing c-Fos immunostaining in the brain and brainstem in male Wistar rats. (B) Digital images representing c-Fos immunostaining in the brain and brainstem in female Wistar rats. (C) Quantification of number of c-Fos-LI cells in male Wistar rats. (D) Quantification of number of c-Fos-LI cells in female Wistar rats. E, *ad lib.* to water (male; n = 3, female; n = 3); D 48 h, water deprivation for 48 h (male; n = 3, female; n = 3); R 12 h, water deprivation for 46 h + *ad lib.* to water for 12 h (male; n = 3, female; n = 3). *$P < 0.05$ versus E and R 12 h. **$P < 0.01$ versus E and R 12 h. Scale bar = 500 μm. OVLT, organum vasculosum of the lamina terminalis; MnPO, median preoptic nucleus; SFO, subfornical organ; SON, supraoptic nucleus; PVN, paraventricular nucleus; AP, area postrema; NTS, nucleus of the solitary tract; RVLM, rostral ventrolateral medulla. Data are presented as the mean ± SEM.

that the system may play an important role in the elicitation of hypovolaemia-induced dipsogenic response (24). The SFO has been described as an integral player in fluid homeostasis, and has been implicated in the *de novo* synthesis of angiotensin II (25). The increases of mRFP1 positive cells in the RVLM may indicate the activation of baroreceptor reflex. It is expected that the blood pressure changed by water deprivation and rehydration, and these may the cause of increases of the mRFP1 positive cells in the RVLM.

In conclusion, we determined the expression patterns of mRFP1 in the transgenic central nervous system after osmotic stimuli. Both acute and chronic osmotic stimulation caused the induction of mRFP1 fluorescence in osmosensitive areas in c-*fos*-mRFP1 transgenic rats. Our results are similar to previous studies of Fos immunostaining (8–10) and it was demonstrated to be of at least equivalent sensitivity compared to native Fos protein detection. Thus, the c-*fos*-mRFP transgenic rats are useful animal model for various physiological studies including the central responses to acute and chronic osmotic challenges. Coupled with deep-brain 'optrode' detection of mRFP1 (26), it may be possible to detect neuronal activation in living, conscious animals.

Acknowledgements

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ORIGINAL PAPER

Effects of food deprivation on the hypothalamic feeding-regulating peptides gene expressions in serotonin depleted rats

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Abstract We examined the effects of serotonin (5-HT) depletion induced by peripheral injection of 5-HT synthesis inhibitor *p*-chlorophenylalanine (PCPA) on the expression of feeding-regulating peptides expressions by using in situ hybridization histochemistry in adult male Wistar rats. PCPA pretreatment had no significant effect on basal levels of *oxytocin*, *corticotropin-releasing hormone* (*CRH*), *thyrotropin-releasing hormone* (*TRH*), *pro-opiomelanocortin* (*POMC*), *cocaine and amphetamine-regulated transcript* (*CART*), *neuropeptide-Y* (*NPY*), *agouti-related protein* (*AgRP*), *melanin-concentrating hormone* (*MCH*) or *orexin* in the hypothalamus. Food deprivation for 48 h caused a significant decrease in *CRH*, *TRH*, *POMC*, and *CART*, and a significant increase in *NPY*, *AgRP* and *MCH*. After PCPA treatment, *POMC* and *CART* did not decrease despite food deprivation. *NPY* was significantly increased by food deprivation with PCPA, but was attenuated compared to food deprivation without PCPA. These results suggest that the serotonergic system in the hypothalamus may be involved in the gene expression of *POMC*, *CART*, and *NPY* related to feeding behavior.

Keywords Serotonin · Feeding · Neuropeptides · Hypothalamus · *p*-chlorophenylalanine

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Introduction

Many physiological behaviors, including emotion, memory, learning, awakening, attention, thermoregulation, and feeding, are manipulated by the serotonergic system [1–4]. With regard to feeding behavior, although its regulation by serotonin (5-HT) is broadly well described, the mechanisms or central pathways that mediate this behavior are still poorly understood. While current approaches to identifying the mechanisms of feeding regulation have focused on feeding-regulating neuropeptides [5], possible interactions between monoamine and neuropeptides in appetite regulation have been clarified [6, 7].

p-chlorophenylalanine (PCPA) depletes brain serotonin (5-HT) by synthetic inhibition [8, 9], of which systemic pretreatment for 2 days resulted in 95 % depletion in hypothalamic serotonin [10]. This pharmaceutical enables us to explicate the interactions between 5-HT and feeding-regulating neuropeptides. Generally, anorexigenic peptides, such as corticotropin-releasing hormone (CRH) [11] and thyrotropin-releasing hormone (TRH) [12, 13], are down-regulated by food deprivation; conversely, orexigenic peptides, such as neuropeptide-Y (NPY) [14] and melanin-concentrating hormone (MCH) [15], are up-regulated. If the feeding-regulating neuropeptides do not

change despite food deprivation after PCPA pretreatment, it can be referred that the neuropeptides may have possible interactions with 5-HT.

Several studies have demonstrated the correlations between 5-HT and hypothalamic feeding-regulating neuropeptides [8, 9, 16–18], although which kind of feeding-regulating peptides are affected by the serotonergic system is unclear. Here, we comprehensively examined the interactions between the serotonergic system and feeding-regulating neuropeptides in the hypothalamus after 48 h food deprivation with or without PCPA pretreatment, using in situ hybridization histochemistry (IHC) in rats.

Materials and methods

Animals

Adult male Wistar rats (180–190 g body weight) were individually housed and maintained in temperature controlled (23–25 °C) conditions under a 12.12 h light/dark cycle (lights on at 0700 hours). All experiments were performed in strict accordance with the guidelines on the use and care of laboratory animals issued by the Physiological Society of Japan, and were approved by the Ethics Committee of Animal Care and Experimentation of University of Occupational and Environmental Health.

Test substance

PCPA (Sigma-Aldrich Japan, Tokyo, Japan) was dissolved in 0.9 % sterile physiological saline (Otsuka Pharmaceutical, Tokyo, Japan) (0.6 mg/1 mL).

Determination of hypothalamic monoamine concentrations

Hypothalamic concentrations of noradrenaline (NA), dopamine (DA), and 5-HT were measured by high-performance liquid chromatography with electrochemical detection (HPLC-ECD). PCPA or saline were administered by intraperitoneally (i.p.) injection daily for 2 days. The rats were decapitated 48 h after second saline ($n = 8$) or PCPA ($n = 8$) treatment followed by removal of the hypothalamus. Briefly, samples were frozen onto dry-ice and stored at −80 °C before the measurements. Samples were homogenized in 0.2 M perchloric acid and centrifuged (8,000*g*) at 4 °C for 30 min. The supernatant was collected and analyzed with HPLC-ECD system (Hitachi, Japan). HPLC-ECD conditions were a modified method of Wetherell et al. [19]. Briefly, separations were performed using a 4.6 × 150 mm ODS C_{18} column. The mobile phase consisted of 0.1 M Na_2PO_4, 0.8 mM OSA, 0.5 M EDTA, and 10 % methanol, and was adjusted to pH 3.63 with phosphoric acid. Column temperature was 40 °C, flow rate 1.0 mL/min, and the detector was set at a potential of +0.75 V relative to an Ag/AgCl reference electrode. The working standard solution was prepared in 0.2 M perchloric acid containing 0.5 mM EDTA and 0.05 mg/mL DHBA was stored at −80 °C.

Experimental procedure

All the rats had ad libitum access to water throughout the experiments. The rats were divided into four groups: saline + ad libitum access to food (SAF, $n = 13$), saline + food deprivation for 48 h (SFD, $n = 13$), PCPA + ad libitum access to food (PAF, $n = 14$), and PCPA + food deprivation for 48 h (PFD, $n = 14$). Saline (10 mL/kg body weight as a single daily dose) or PCPA (200 mg/10 mL/kg body weight as a single daily dose) was administered i.p. at day 0 and day 1 (0900–1000 hours). After the administration of saline or PCPA at day 1, food was deprived in SFD and PFD. Body weights and food intake in all the experimental groups were measured every 24 h from day 1 to day 6.

On day 3, some of those rats (SAF, $n = 7$, SFD, $n = 7$, PAF, $n = 8$, PFD, $n = 8$) were decapitated immediately after the treatment without being anesthetized, followed by prompt removal of the brain onto dry ice, then storing at −80 °C. Trunk blood samples were taken during decapitation, and were collected into chilled reaction tubes (Greiner Bio-One) containing an aprotinin/EDTA mixture. The blood samples were Immediately,centrifuged for 10 min at 4 °C, 1,000*g*, after which, a 15-μL sample of plasma was taken for measuring plasma osmolality (P-Osm) using a ONE-TEN osmometer (FISKE, Norwood, MA, USA), 500 μL for plasma 5-HT (SRL, Tokyo, Japan), 500 μL for plasma leptin (SRL), 1,000 μL for plasma active and desacyl ghrelin (SRL), and 10 μL for measuring plasma glucose (PG) using a Medisafe Reader GR-101 (Terumo, Tokyo, Japan). Finally, 100 μL of 1 M HCl was added to each tube for measuring active and desacyl ghrelin in order to protect against decomposition.

In situ hybridization histochemistry

The removed brains were cut into 12-μm slices and thaw-mounted on gelatin/chrome alum-coated slides. The locations of the hypothalamic areas, including the supraoptic nucleus (SON), paraventricular nucleus (PVN), arcuate nucleus (ARC), and lateral hypothalamic area (LHA), were determined according to coordinates of the rat brain atlas.

^{35}S 3′- end-labeled deoxyoligonucleotide complementary to transcripts encoding *oxytocin*, *CRH*, *TRH*, *pro-opiomelanocortin* (*POMC*), *cocaine and amphetamine-regulated transcript* (*CART*), *NPY*, *agouti-related protein* (*AgRP*), *MCH*, and *orexin* were used (*oxytocin* probe sequence, 5′-CTC GGA GAA GGC AGA CTC AGG GTC GCA GGC-3′; *CRH* probe sequence, 5′-CAG TTT CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GGC-3′; *TRH* probe sequence, 5′-GTC TTT TTC CTC CTC CTC CCT TTT GCC TGG ATG CTG CGC TTT TGT GAT-3′; *POMC* probe sequence, 5′-TGG CTG CTC TCC AGG CAC CAG CTC CAC ACA TCT ATG GAG G-3′; *CART* probe sequence, 5′-TCC TTC TCG TGG GAC GCA TCA TCC ACG GCA GAG TAG ATG TCC AGG-3′; *NPY* probe sequence, 5′-CAA ATG GAT GAT TGG TCA TTT CAA CAT AGA GTT GGG GGC TTG CT-3′; *AgRP* probe sequence, 5′-CGA CGC GGA GAA CGA GAC TCG CGG TTC TGT GGA TCT AGC ACC TCT GCC-3′; *MCH* probe sequence, 5′-CCA ACA GGG TCG GTA GAC TCG TCC CAG CAT-3′; and *orexin* probe sequence, 5′-TCC TCA TAG TCT GGA GGC AGG TGG AAG GGT TCC CCA CTG CTA GTG-3′) The specificity of these probes were confirmed by previous studies [20, 21].

The probe was 3′-end-labeled using terminal deoxynucleotidyl transferase and [^{35}S] dATP. The in situ hybridization protocol has been previously described in detail [22]. Briefly, sections were fixed in 4 % (w/v) formaldehyde for 5 min and incubated in saline containing 0.25 % (v/v) acetic anhydride and 0.1 M triethanolamine for 10 min and then dehydrated, delipidated in chloroform, and partially rehydrated. Hybridization was carried out overnight at 37 °C in 45 μL of hybridization buffer under a Nescofilm (Bando Kagaku, Osaka, Japan) cover slip. A total count of 1×10^5 c.p.m. for the *oxytocin* transcripts and 1×10^6 c.p.m. for the *CRH*, *TRH*, *POMC*, *CART*, *NPY*, *AgRP*, *MCH* and *orexin* transcripts were used per slide. After hybridization, the sections were washed 4 times with SSC (150 mM NaCl and 15 mM sodium citrate) for 1 h at 55 °C and for an additional hour with two changes of SSC at room temperature. The hybridized sections containing hypothalamus were exposed for autoradiography (Hyperfilm; Amersham, Bucks, UK) for 6 h for the *oxytocin* probe, 5 days for the *MCH* and *orexin* probe, and 1 week for the *CRH*, *TRH*, *POMC*, *CART*, *NPY*, and *AgRP* probe. The resulting images were analyzed by computerized densitometry using a MCID imaging analyzer (Imaging Research, Ontario, Canada). The mean optical densities (OD) of the autoradiographs were measured by comparison with simultaneously exposed ^{14}C-labeled microscale samples (Amersham) and represented in arbitrary units setting the mean OD obtained from control rats.

Statistical analysis

The mean ± SEM was calculated from the results of the change in body weight, cumulative food intake, plasma measurements, and ISH studies. In the results of ISH, the expression levels of the genes were expressed as a percentage of PAF. All data were analyzed by one-way ANOVA followed by a Bonferroni-type adjustment for multiple comparisons (Origin Pro v.8.5 J; Lightstone, Tokyo, Japan). Statistical significance was set at $P < 0.05$.

Results

Hypothalamic monoamine concentrations after PCPA treatment

Hypothalamic NA, DA, and 5-HT were measured using HPLC (Fig. 1). No statistically differences were observed in hypothalamic NA and DA; however, hypothalamic 5-HT levels nearly depleted in PCPA-treated group compared to saline-treated group (Fig. 1).

Changes in body weight

The body weight of each group was measured from day 1 to day 6 (Fig. 2). The body weight gradually increased during the experiments in SAF (Fig. 2). The body weight in PAF gradually increased after day 1, but was statistically different compared to SAF after day 1. The body weight decreased after PCPA administration (day 1) in PFD and PAF compared to SAF (Fig. 2). A decrease in body weight was observed in SFD and PFD after starting food

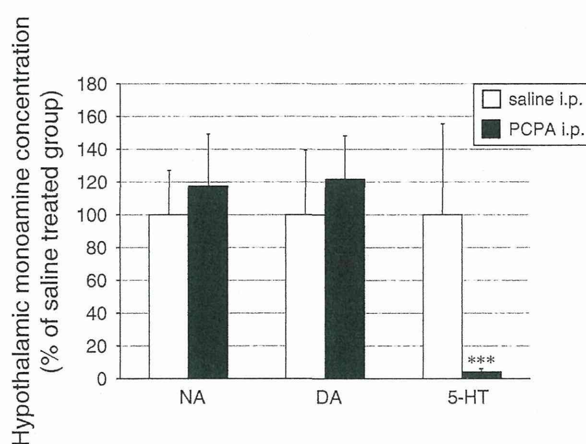

Fig. 1 Hypothalamic monoamine concentration after i.p. administration of saline or PCPA. These data were measured by HPLC. The amount of each monoamine level represents % from saline group. Data are presented as mean ± SEM. $***P < 0.001$ vs. saline group. *NA* noradrenaline, *DA* dopamine, *5-HT* serotonin

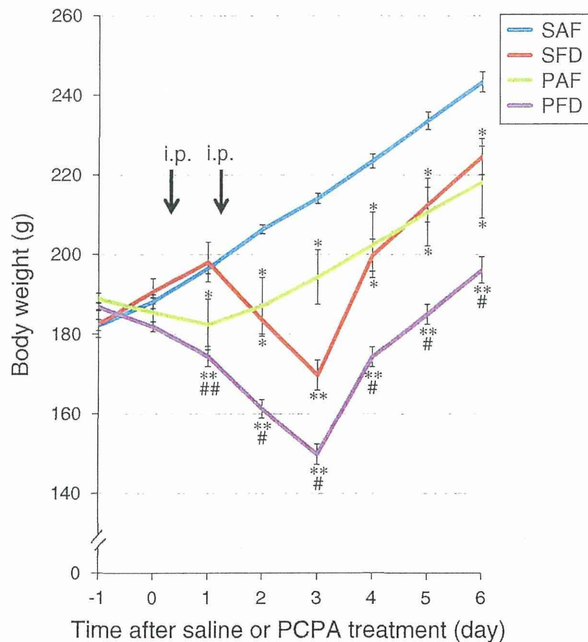

Fig. 2 Changes in body weight changes in body weight from day 1 to day 6. *Arrows* indicate saline or PCPA i.p. administration. Data are presented as mean ± SEM. $^{*}P < 0.05$ vs. SAF, $^{**}P < 0.01$ vs. SAF and PAF, $^{\#}P < 0.05$ vs. SFD, $^{\#\#}P < 0.01$ vs. SFD. *SAF* saline + ad libitum access to food, *SFD* saline + food deprivation for 48 h, *PAF* PCPA + ad libitum access to food, *PFD* PCPA + food deprivation for 48 h

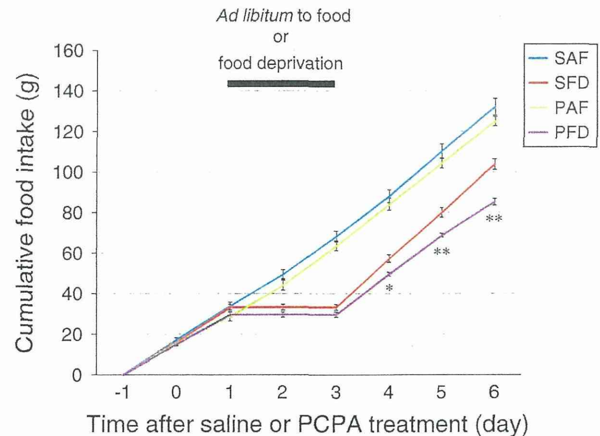

Fig. 3 Changes in food intake Cumulative food intake (g) from day 1 to day 6. Data are presented as mean ± SEM. $^{*}P < 0.05$ vs. SFD, $^{**}P < 0.01$ vs. PFD. Saline or PCPA was i.p. administered at day 0 and day 1

deprivation (Fig. 2). There were no statistical differences between the body weight of SFD and PAF after day 4.

Food intake

Cumulative food intake during the experiment was measured from day 1 to day,6 (Fig. 3). Cumulative food intake was comparable in all among the groups at day 0 and day 1. Cumulative food intake in SFD and PFD was null after starting food deprivation (Fig. 3). Cumulative food intake in PFD decreased at days 4, 5, and 6 compared to SFD (Fig. 3).

Plasma measurement

PG, P-Osm, 5-HT, leptin, and active/desacyl ghrelin concentration at day 3 were measured. PG in SFD (62 ± 2.4 mg/dL) and PFD (75 ± 4.1 mg/dL) decreased significantly compared to that in SAF (108 ± 1.8 mg/dL) and PAF (105 ± 3.9 mg/dL). No statistical differences were seen between SAF and PAF or between SFD and PFD. There were no significant differences in P-Osm among all the experimental groups (SAF: 311 ± 1.3 mOsm/kg; SFD: 310 ± 2.78 mOsm/kg; PAF: 307 ± 2.2 mOsm/kg; PFD: 310 ± 1.7 mOsm/kg). Plasma 5-HT level significantly decreased in SFD (384 ± 19.4 ng/mL) compared to SAF (532 ± 42.0 ng/mL) (Fig. 4a). That in PAF (75 ± 14.3 ng/mL) and PFD (35 ± 10.3 ng/mL) was almost depleted compared to SAF and SFD (Fig. 4a). It also significantly decreased in PFD compared to PAF (Fig. 4a). Plasma leptin level in SFD (1.5 ± 0.1 ng/mL) and PFD (1.7 ± 0.1 ng/mL) significantly decreased compared to SAF (3.9 ± 0.3 ng/mL) and PAF (3.4 ± 0.5 ng/mL) (Fig. 4b). No statistical differences were observed between SAF and PAF or SFD and PFD. Plasma desacyl ghrelin in SFD (793.3 ± 83.0 fmol/mL) and PFD (751.6 ± 72.0 fmol/mL) significantly increased compared to those of SAF (213.3 ± 32.9 fmol/mL) and PAF (344 ± 48.6 fmol/mL) (Fig. 4c). There were statistical differences between SAF and PAF (Fig. 4c). Plasma active ghrelin increased in SFD (20.2 ± 6.2 fmol/mL) compared to SAF (5.3 ± 0.6 fmol/mL) (Fig. 4d). Those in PAF (53.0 ± 11.6 fmol/mL) and PFD (65.8 ± 5.8 fmol/mL) significantly increased compared to SAF and SFD (Fig. 4d). The ratio of plasma active/desacyl ghrelin $\times 10^2$ in PAF (15.3 ± 1.9) and PFD (9.1 ± 0.9) significantly increased compared to those in SAF (2.6 ± 0.3) and SFD (2.5 ± 0.6) (Fig. 4e). There were statistical differences between PAF and PFD (Fig. 4e).

Feeding-regulating peptides in the SON and the PVN

The feeding-regulating peptides in the SON and the PVN were measured by ISH followed by quantification using MCID. The gene expression of the *oxytocin* in the SON and the PVN was comparable among all the experimental groups (Fig. 5a A-a–A-d, B-a–B-d, b). The gene expression of the *CRH* and the *TRH* in the PVN in SFD (Fig. 5a C-b, D-b, b) and PFD (Fig. 5a D-b, D-d, b) decreased significantly compared to those in SAF (Fig. 5a C-a, D-a,

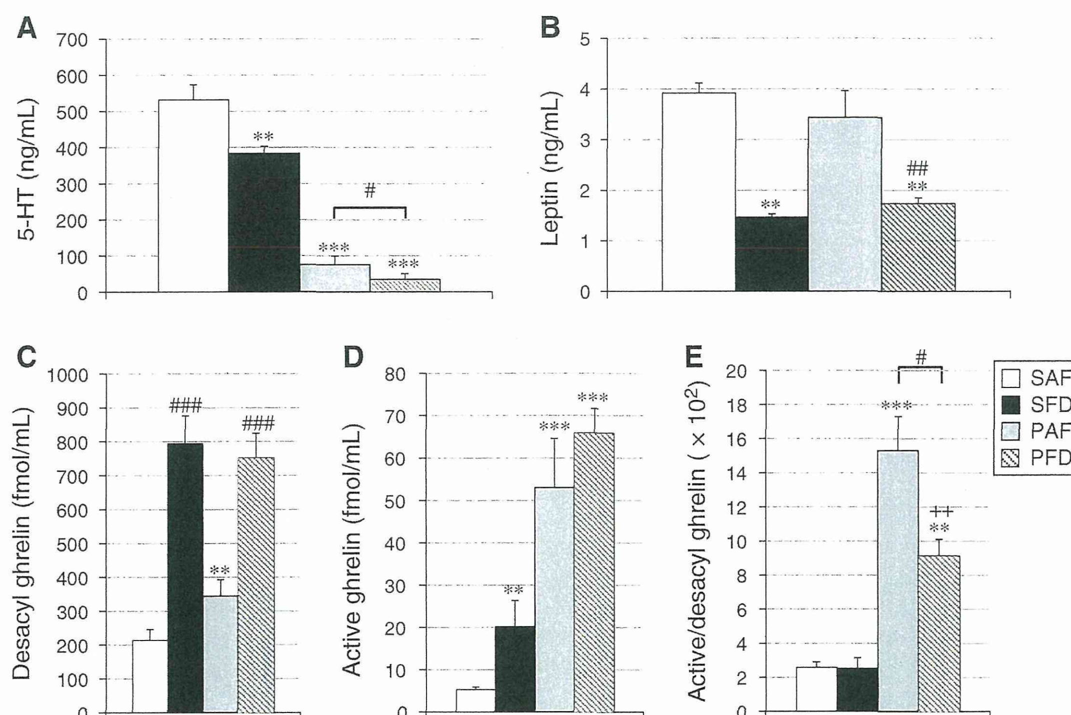

Fig. 4 Plasma concentrations of 5-HT, leptin, and active/desacyl ghrelin ratio Plasma levels of 5-HT (**a**), leptin (**b**), desacyl ghrelin (**c**), active ghrelin (**d**), and the ration of active/desacyl ghrelin (**e**) are shown. Data are presented as mean ± SEM. $^{**}P < 0.01$ vs. SAF $^{***}P < 0.001$ vs. SAF and SFD, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. PAF, $^{\#\#\#}P < 0.001$ vs. SAF and SFD, $^{++}P < 0.01$ vs. SFD

b) and PAF (Fig. 5a C–c, D-c, b). There were no statistical differences between SAF and PAF, or between SFD and PFD in the gene expression of the *oxytocin*, *CRH*, and *TRH*.

Feeding-regulating peptides in the ARC and the LHA

The feeding-regulating peptides in the ARC and the LHA were measured by ISH followed by quantification using MCID. The gene expression of the *POMC* and the *CART* in the ARC in SFD (Fig. 6a A-b, B-b, and b) decreased significantly compared to SAF (Fig. 6a A-a, B-a, b), whereas those in PFD (Fig. 6a A-d, B-d, b) were comparable to PAF (Fig. 6a A-c, B-c, b). The gene expression of the *NPY* and *AgRP* in the ARC in SFD (Fig. 6a C-b, D-b, b) and PFD (Fig. 6a C-d, D-d, b) increased significantly compared to SAF (Fig. 6a C-a, D-a, b) and PAF (Fig. 6a C–c, D-c, b). With regard to the gene expression of the *AgRP*, there were no significant differences between SFD (Fig. 6a D-b, b) and PFD (Fig. 6a D-d, b). However, the gene expression of the *NPY* in SFD (Fig. 6a C-b, b) and PFD (Fig. 6a C-d, b) differed significantly.

The gene expression of the *MCH* in the LHA in SFD (Fig. 6a E-b, b) and PFD (Fig. 6a E-d, b) increased significantly compared to those in SAF (Fig. 6a E-a, b) and PAF (Fig. 6a E-c, b). The gene expression of the *MCH* did not differ between SFD and PFD.

The gene expression of the *orexin* was comparable among all the experimental groups (Fig. 6a F-a–F-d, b). No significant differences were observed between SAF and PAF in the gene expression of the *POMC*, *CART*, *NPY*, *AgRP*, *MCH*, and *orexin*.

Discussion

The present study comprehensively showed the relationships between the serotonergic system and feeding-regulating peptides in the hypothalamus. These results imply that the gene expression of the *POMC*, *CART* and *NPY* in the ARC may be involved in the serotonergic system in the hypothalamus.

After the PCPA treatment, hypothalamic contents of NA, DA, and 5-HT were measured by HPLC to confirm the depletion of 5-HT after the treatment. As shown in Fig. 1, although NA and DA did not change with or without PCPA, 5-HT level nearly depleted after PCPA treatment, which was consistent with a previous report [10]. We made sure that the 5-HT level in the hypothalamus definitely depleted after the PCPA treatment.